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Report

Sequential Protein Recruitment in *C. elegans* Centriole Formation

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Summary

Formation of the microtubule-based centriole is a poorly understood process that is crucial for duplication of the centrosome, the principal microtubule-organizing center of animal cells [1]. Five proteins have been identified as being essential for centriole formation in *Caenorhabditis elegans*: the kinase ZYG-1, as well as the coiled-coil proteins SAS-4, SAS-5, SAS-6, and SPD-2 [2–9]. The relationship between these proteins is incompletely understood, limiting understanding of how they contribute to centriole formation. In this study, we established the order in which these five proteins are recruited to centrioles, and we conducted molecular epistasis experiments expanding on earlier work. We find that SPD-2 is loaded first and is needed for the centriolar localization of the four other proteins. ZYG-1 recruitment is required thereafter for the remaining three proteins to localize to centrioles. SAS-5 and SAS-6 are recruited next and are needed for the presence of SAS-4, which is incorporated last. Our results indicate in addition that the presence of SAS-5 and SAS-6 allows diminution of centriolar ZYG-1. Moreover, astral microtubules appear dispensable for the centriolar recruitment of all five proteins. Several of these proteins have homologs in other metazoans, and we expect the assembly pathway that stems from our work to be conserved.

Results

Sequential Recruitment of Centriolar Proteins in *C. elegans* Embryos

Centrioles are minute cylindrical structures that contain nine sets of microtubules arranged in a radial fashion [10]. At the onset of the centrosome duplication cycle, the two tightly apposed centrioles split slightly from one another. Each of these mother centrioles then seeds formation of a daughter centriole. Centriole formation has been described by ultrastructural analysis in vertebrate cells that primarily monitored the growth of the microtubules, which constitute the defining feature of centrioles [11, 12]. By contrast, the molecular tenets of this assembly process have remained elusive.

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The *C. elegans* embryo has proven well suited for investigating centrosome duplication [13]. The five proteins known to be essential for centriole formation in this organism are enriched at centrioles and present at lower levels in the cytoplasm of early embryos. ZYG-1 is found at centrioles primarily during anaphase [2], whereas the four other proteins are centriolar throughout the cell cycle [3–9]. Furthermore, SPD-2 is enriched in the PCM compared to the cytoplasm, possibly reflecting its additional role in PCM assembly [5, 6].

We set out to address the order in which ZYG-1, SAS-4, SAS-5, SAS-6, and SPD-2 are recruited to centrioles. We designed experiments that distinguish de novo centriolar recruitment from the prior presence of proteins at centrioles. Such experiments are rendered possible because the sperm contributes the sole pair of centrioles to the newly fertilized embryo. These two centrioles split slightly from one another, and each seeds the formation of a daughter centriole. Because the initial pair of centrioles is of paternal origin, one can assay specifically centriolar recruitment or exchange that occurs in the one-cell-stage embryo, provided the centrioles contributed by the sperm do not harbor the protein under scrutiny.

We first analyzed SPD-2. Because GFP-SPD-2 is not present in sperm, in contrast to the endogenous protein [5, 6], we determined the time at which GFP-SPD-2 is first detected at centrioles in one-cell-stage embryos. We used double labeling with antibodies against SAS-4 to mark all centrioles and against GFP to detect GFP-SPD-2 recruitment. As shown in Figure 1A, we found that GFP-SPD-2 is first detected at centrioles during meiosis I (13 of 18 embryos). Even though endogenous SPD-2 is present in sperm, it is lost rapidly after fertilization in embryos depleted of SPD-2 [6]. Therefore, we also assayed recruitment of the endogenous protein and found that, as for GFP-SPD-2, SPD-2 is first detected at centrioles during meiosis I (data not shown). Endogenous ZYG-1 is not present in sperm [2], which enabled us to similarly assay its recruitment after fertilization. We found that ZYG-1 is also first detected at centrioles during meiosis I (Figure 1E; 8 of 22 embryos). In conducting these experiments, we noted that the paternally contributed centrioles can be first distinguished a single entities during meiosis II, indicating that splitting has occurred by that time (see Figure S1 in the Supplemental Data available with this article online). Overall, we conclude that SPD-2 and ZYG-1 centriolar recruitment initiates as early as meiosis I, prior to when splitting of the centriole pair can be observed by light microscopy.

We next examined SAS-5. In this case, both the endogenous protein and GFP-SAS-5 are present in sperm centrioles [7]. Therefore, we performed marked mating experiments by crossing hermaphrodites expressing GFP-SAS-5 to wild-type males, the sperm of which provide centrioles not carrying the fusion protein. By contrast to the situation with SPD-2 and ZYG-1, we found that GFP-SAS-5 is not present at centrioles during

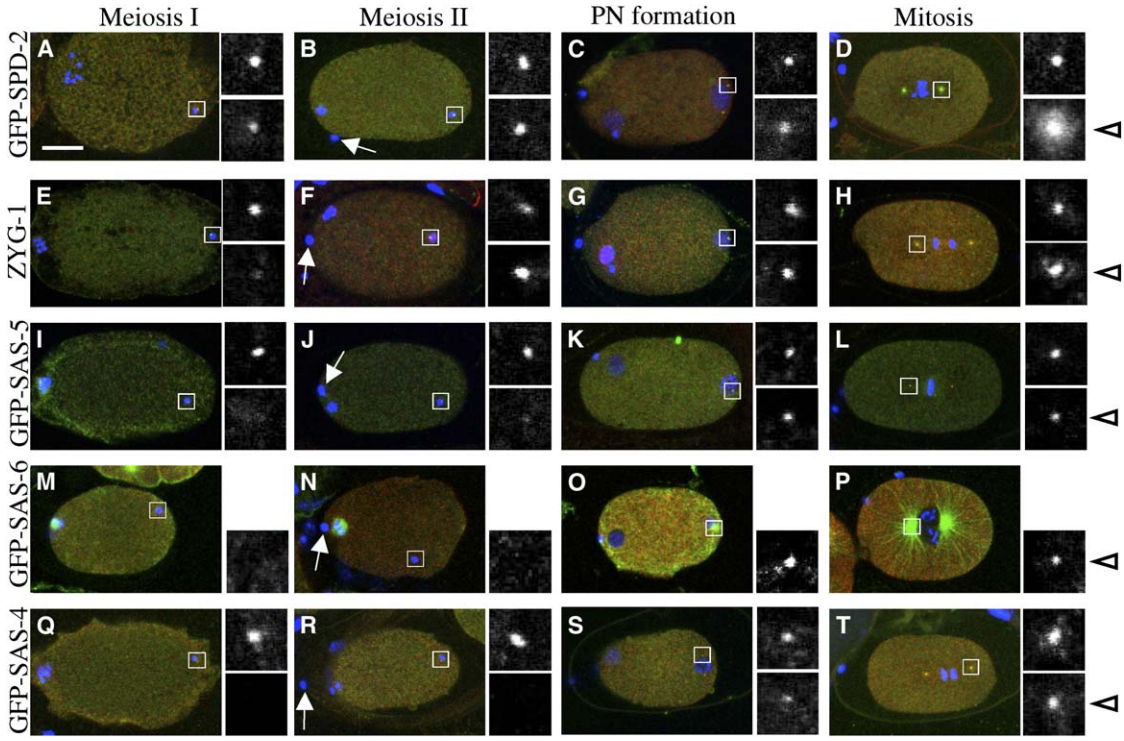

Figure 1. Sequential Recruitment of Centriolar Proteins in *C. elegans* Embryos

One-cell-stage embryos during meiosis I, meiosis II, pronuclear (PN) formation (corresponding to early S phase), and mitosis, as indicated. In all figures, anterior is to the left, insets are approximately 6-fold magnified views of centrosomes, arrowheads indicate the focus of the panels where appropriate, and scale bars represent 10 μm; moreover, DNA is shown in blue. Arrows in Figures 1B, 1F, 1J, 1N, and 1R point to the first polar body.

(A–D) GFP-SPD-2 transgenic embryos stained with antibodies against SAS-4 (red and top insets) and GFP (green and bottom insets). Thirteen of 18 meiosis I embryos and 6 of 6 meiosis II embryos exhibited GFP-SPD-2 signal at centrioles; the signal was typically weaker in meiosis I (compare [A] and [B]). Note that GFP-SPD-2 is also present at the PCM during mitosis [5, 6] (also see Figure 2).

(E–H) GFP-SAS-4 transgenic embryos stained with antibodies against GFP (green and top insets) and ZYG-1 (red and bottom insets). Eight of 22 meiosis I embryos and 13 of 17 meiosis II embryos exhibited ZYG-1 signal at centrioles; the signal was typically weaker in meiosis I (compare [E] and [F]).

(I–L) Embryo coming from an oocyte expressing GFP-SAS-5, fertilized by wild-type sperm and stained with antibodies against SAS-4 (red and top insets) and GFP (green and bottom insets). Zero of 15 meiosis I embryos and five of five meiosis II embryos exhibited GFP-SAS-5 signal at centrioles.

(M–P) GFP-SAS-6 transgenic embryos stained with antibodies against α-tubulin (green) and GFP (red and insets). Note that double labeling with rabbit antibodies against GFP and mouse antibodies against α-tubulin was conducted in this instance; this ensured more sensitive detection of the GFP-SAS-6 signal, which was weaker than that of the other fusion proteins.

(Q–T) Embryo coming from an oocyte expressing GFP-SAS-4, fertilized by wild-type sperm, and stained with antibodies against SAS-4 (red and top insets) and GFP (green and bottom insets).

meiosis I (Figure 1I; 15 of 15 embryos). Instead, GFP-SAS-5 is first detected weakly at the end of meiosis II (Figure 1J), with the centriolar signal becoming more robust thereafter (Figures 1K and 1L). Because GFP-SAS-6 is not present in sperm [9], in contrast to the endogenous protein, we could simply assess when the fusion protein is first recruited to centrioles after fertilization. As shown in Figures 1M–1P, we found that centriolar GFP-SAS-6 is first detected shortly after meiosis II and more robustly so thereafter (Figures 1M–1P), much like GFP-SAS-5. This is in line with the fact that SAS-5 and SAS-6 physically interact and are mutually dependent for their centriolar localization [9]. Overall, we conclude that SAS-5 and SAS-6 are recruited after SPD-2 and ZYG-1.

Because both SAS-4 and GFP-SAS-4 are present in sperm centrioles [3, 4], we also conducted marked mating experiments to investigate GFP-SAS-4 centriolar recruitment in one-cell-stage embryos. As reported [8], we found that GFP-SAS-4 is incorporated progressively to centrioles during the first cell cycle, starting at the time of pronuclear formation (Figures 1Q–1T). Taken together, these observations establish the following temporal sequence of recruitment to centrioles: first, SPD-2 and ZYG-1; second, SAS-5 and SAS-6; and third, SAS-4.

SPD-2 Controls Centriolar Recruitment of ZYG-1, SAS-5, SAS-6, and SAS-4

We next addressed whether this temporal sequence corresponds to related episatic interactions. In one extreme scenario, the five proteins could be recruited independently of one another. Alternatively, the proteins that are recruited early in the sequence may be needed for the presence of some that are recruited later. We initially investigated whether SPD-2 is required for the

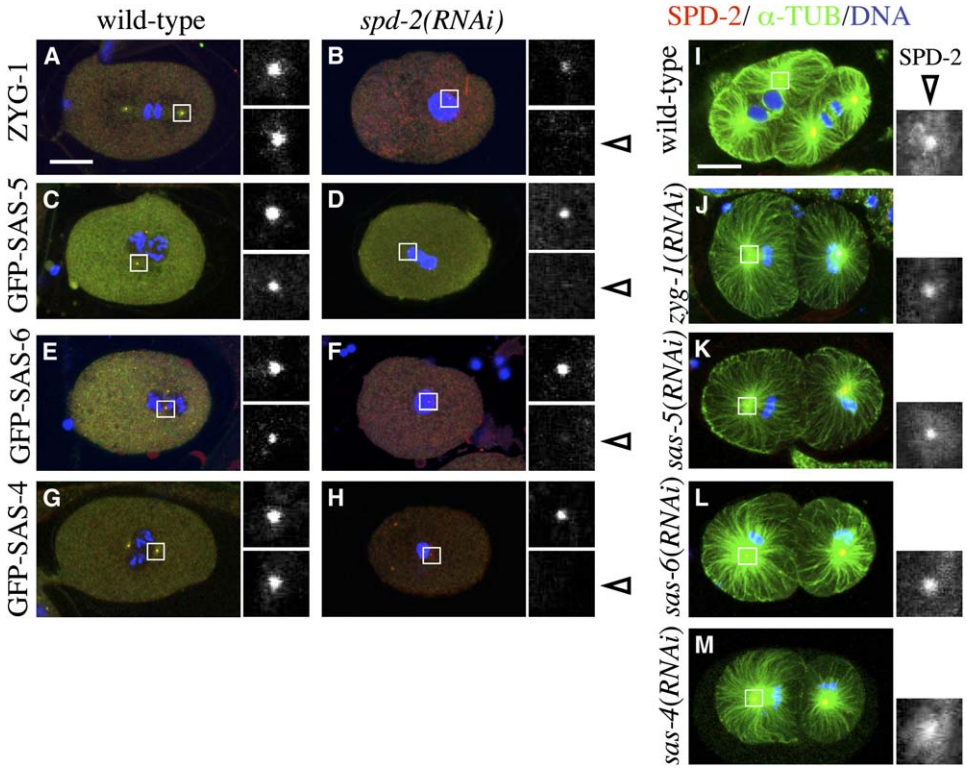

Figure 2. Reciprocal Relationship between SPD-2 and the Four Other Proteins

(A–H) One-cell-stage embryos either not subjected (left column) or subjected (right column) to *spd-2*(*RNAi*). (A and B) GFP-SAS-4 transgenic embryos stained with antibodies against GFP (green and top insets) and ZYG-1 (red and bottom insets). (C and D) GFP-SAS-5 transgenic embryos stained with antibodies against SAS-4 (red and top insets) and GFP (green and bottom insets). (E and F) GFP-SAS-6 transgenic embryos stained with antibodies against SAS-4 (red and top insets) and GFP (green and bottom insets). (G and H) Embryo coming from an oocyte expressing GFP-SAS-4, fertilized by wild-type sperm and stained with antibodies against SAS-4 (red and top insets) and GFP (green and bottom insets). Note that DNA condensation is abnormal in *spd-2*(*RNAi*) embryos [19]. Note also that levels of SAS-4 or GFP-SAS-4 (top insets) on paternally contributed centrioles are reduced in all cases compared to the wild-type.

(I–M) Embryos of the indicated genotypes in the second cell cycle stained with antibodies against SPD-2 (red) and α-tubulin (green). The insets show the SPD-2 signal alone. Note that SPD-2 is present both at centrioles and to a lesser extent in the surrounding PCM in all cases. Note also that the signal in the PCM increases during mitosis [5, 6].

centriolar recruitment of the other four proteins. As illustrated in Figures 2A–2H, we found that ZYG-1, GFP-SAS-5, GFP-SAS-6, and GFP-SAS-4 all fail to be recruited to centrioles in *spd-2*(*RNAi*) embryos. Moreover, we found that levels of SAS-4 on paternally contributed centrioles are diminished in *spd-2*(*RNAi*) embryos compared to the wild-type (Figures 2B–2H, top insets), as suggested by previous observations [5, 6]. Because SAS-4 is stably associated with the centriole in the wild-type [3, 4], this indicates that SPD-2 also plays a role in maintaining SAS-4 after its incorporation into centrioles. In a converse set of experiments, we found that SPD-2 distribution is not altered in *zyg-1*(*RNAi*), *sas-5*(*RNAi*), *sas-6*(*RNAi*), or *sas-4*(*RNAi*) embryos (Figures 2I–2M). Overall, we conclude that SPD-2 controls centriolar recruitment of the four other proteins.

Diminution of Centriolar ZYG-1 Requires SAS-5 and SAS-6

We next investigated ZYG-1, which is required for the presence of centriolar SAS-5 and SAS-6, which are themselves needed for GFP-SAS-4 recruitment [7–9]. In a converse set of experiments, we examined ZYG-1 distribution in embryos compromised for SAS-5, SAS-6, or SAS-4 function. In the wild-type, levels of ZYG-1 at centrioles are regulated across the cell cycle, with the signal being minimal during interphase and maximal during anaphase (Figures 3A and 3B and Figure S2) [2]. We found that ZYG-1 still localizes to centrioles in *sas-5*(*RNAi*) embryos (data not shown) as well as in *sas-5*(*t2079*) mutant embryos (Figures 3C and 3D), in which SAS-5 and SAS-6 are not present at centrioles [7, 9]. ZYG-1 also localizes to centrioles in *sas-6*(*RNAi*) and *sas-4*(*RNAi*) embryos (Figures 3E–3H). Together, these observations establish that ZYG-1 acts upstream of SAS-5 and SAS-6 centriolar recruitment, which themselves act upstream of SAS-4 centriolar recruitment.

We discovered in the course of these experiments that ZYG-1 levels at centrioles remain high throughout the cell cycle in *sas-5*(*t2079*) mutant embryos and *sas-6*(*RNAi*) embryos (Figures 3C–3F, compare with Figures 3A and 3B). By contrast, levels of centriolar ZYG-1 still oscillate across the cell cycle in *sas-4*(*RNAi*) embryos, with the signal being minimal during interphase and maximal during anaphase (Figures 3G and 3H). Together, these results indicate that SAS-5 and SAS-6 are required for the diminution of centriolar ZYG-1 during interphase. Because SAS-5 and SAS-6 are present

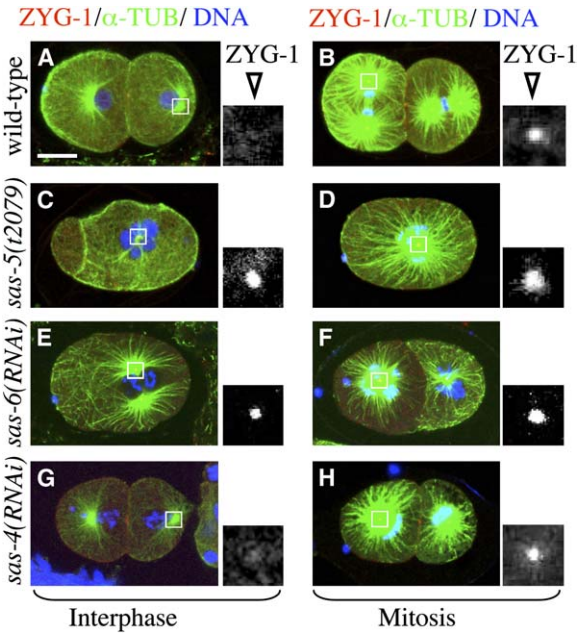

Figure 3. Diminution of Centriolar ZYG-1 Requires SAS-5 and SAS-6

Embryos of the indicated genotypes in the first (D and E) or second (A–C, F–H) cell cycle, either in interphase or in mitosis, as indicated, stained with antibodies against ZYG-1 (red) and α-tubulin (green). The insets show the ZYG-1 signal alone. Note that, whereas ZYG-1 is barely detected at centrioles during interphase in the wild-type or in *sas-4*(*RNAi*) embryos, it is clearly present in *sas-5*(*t2079*) and *sas-6*(*RNAi*) embryos. Note also that, although different stages of interphase are shown, ZYG-1 levels are low throughout interphase in the wild-type (see Figure S2).

in the cytoplasm but absent from centrioles in *sas-5*(*t2079*) mutant embryos [7, 9], these results suggest in addition that this requirement reflects the presence or activity of centriolar SAS-5 and SAS-6.

Microtubules and Recruitment of Centriolar Proteins

We wished to place the recruitment of centriolar microtubules in the pathway that emerges from our study. However, we could not assay their recruitment using GFP-β-tubulin, because the fusion protein is also incorporated in the remainder of the microtubule cytoskeleton, masking the specific centriolar signal (data not shown). Therefore, we do not know the timing of centriolar microtubule recruitment relative to the five proteins discussed above. Nevertheless, we set out to test whether astral microtubules are required for the recruitment of these proteins using RNAi against the α-tubulin gene *tba-2* [14].

In severely affected *tba-2*(*RNAi*) embryos, tubulin is detected only in paternally contributed centrioles and their immediate vicinity, as expected from the fact that RNAi does not target sperm under these experimental conditions (Figures 4B, 4D, and 4F) [15]. Interestingly, we observed that the two paternally contributed centrioles split from one another in one-cell-stage *tba-2*(*RNAi*) embryos (Figures 4B and 4F). Therefore, astral microtubules do not appear to be needed for centriole splitting at the onset of the duplication cycle in *C. elegans* embryos, as in vertebrate somatic cells [16]. We noted also that there are only two centrosomes in *tba-2*(*RNAi*) embryos, even after several cell cycles (data not shown). In principle, these two centrosomes could each contain a pair of centrioles if just one round of centriole formation had occurred. However, since centrioles can split from one another in *tba-2*(*RNAi*) embryos, four centrosomes, each containing one centriole, would be expected in this scenario. As only two centrosomes are present, it appears instead that completion of daughter centriole formation is impaired and that each centrosome contains only one paternally contributed centriole in *tba-2*(*RNAi*) embryos. Therefore, centriole formation does not seem to occur in *tba-2*(*RNAi*) embryos. Similarly, centriole formation fails in vertebrate somatic cells treated with high doses of colcemid [16].

We reported previously that GFP-SAS-5 and GFP-SAS-6 are recruited to centrioles in *tba-2*(*RNAi*) embryos [7, 9]. We found the same to be true for GFP-SPD-2 (data not shown), as well as SPD-2, ZYG-1, and GFP-SAS-4 (Figure 4B, 4D, and 4F). Although we cannot exclude the possibility that residual tubulin contributes to the recruitment of these proteins, these results strongly suggest that SPD-2, ZYG-1, SAS-5, SAS-6, and SAS-4 can all be recruited independently of astral microtubules.

Discussion

Daughter centriole formation has been described by ultrastructural studies as occurring in a gradual fashion, orthogonal to the mother centriole [11, 12]. Whereas other assembly processes, such as bacteriophage T4 tail formation or budding yeast spindle pole body duplication, exhibit a series of discrete intermediates recognizable by electron microscopy [17, 18], this does not appear to be the case for centriole formation, apart from the gradual elongation of the characteristic microtubule-based structure. Thus, it was not clear whether this particular assembly process could also be described as a series of consecutive steps.

In this study, we provide evidence for an emerging pathway for centriole formation in *C. elegans*. Together with earlier work, our findings lead us to propose the following sequence of events (Figures 4G and 4H). First, SPD-2 is recruited to each mother centriole or to a closely associated structure. SPD-2 is needed for the centriolar recruitment of ZYG-1, which in turn is required for the remaining three proteins to localize to centrioles. SAS-5 and SAS-6 are recruited next and are needed for SAS-4 to be incorporated thereafter. Furthermore, our results suggest that assembly of centriolar microtubules occurs downstream of SAS-4 incorporation or in parallel to the entire pathway. In addition, the PCM components SPD-5 and γ-tubulin play a partially redundant role in centriole formation [8], and it will be interesting to investigate their placement in this sequence. Overall, we conclude that, like other assembly processes, centriole formation can be described as a series of consecutive steps that entails the sequential recruitment of at least five proteins that ensure formation of a daughter centriole next to each mother centriole once per cell cycle.

SPD-2 is unique among the five proteins investigated in being also required for PCM assembly [5, 6, 19]. In embryos depleted of SPD-2, the coiled-coil protein SPD-5 is not recruited to centrosomes, resulting in the absence of other PCM components, including the Aurora kinase

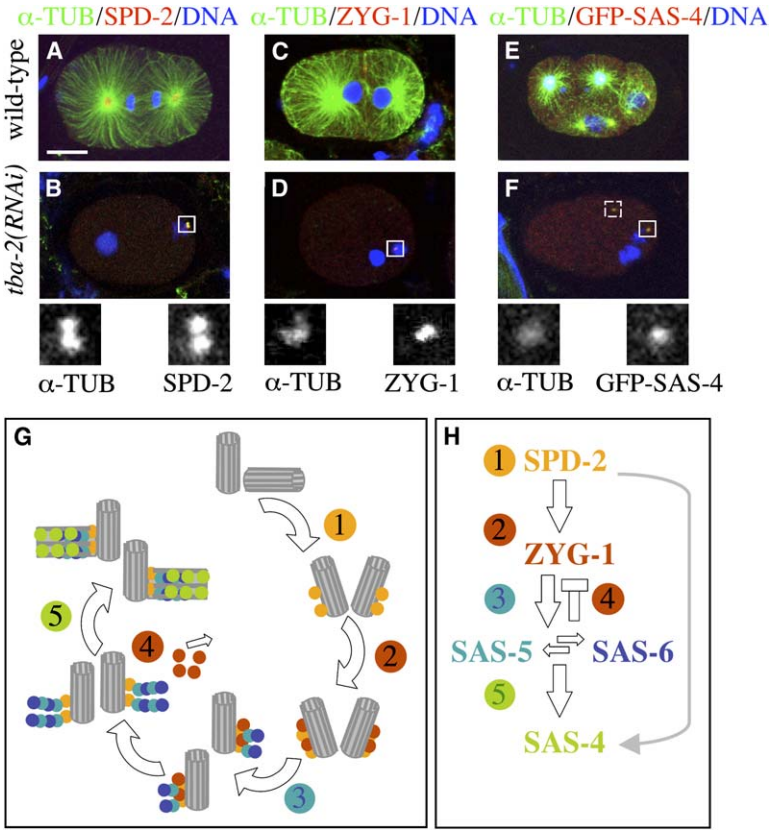

Figure 4. Astral Microtubules Are Dispensable for Centriolar Recruitment of SPD-2, ZYG-1, and SAS-4; Working Model for Centriole Formation

(A–D) Wild-type (A and C) or *tba-2*(*RNAi*) (B and D) embryos in the first cell cycle ([A], anaphase; [B], prophase; [C], telophase; [D], pronuclear formation) stained with antibodies against α-tubulin (green) and either SPD-2 (A and B) or ZYG-1 (C and D) (both red). The insets show the SPD-2 or the ZYG-1 signal alone in *tba-2*(*RNAi*) embryos. Note that splitting of centrioles is not apparent in (D), most likely because the two centrioles are too close to one another at this early stage of the cell cycle.

(E and F) Embryos coming from an oocyte expressing GFP-SAS-4, fertilized by wild-type sperm and either not treated ([E], four-cell stage) or subjected to *tba-2*(*RNAi*) ([F], one-cell stage, mitosis). The embryos were stained with antibodies against α-tubulin (green) and GFP (red). The insets show the α-tubulin and the GFP-SAS-4 signal alone in the *tba-2*(*RNAi*) embryo. The stippled square in the low magnification view of the *tba-2*(*RNAi*) embryo indicates the second centrosome.

(G) Proposed succession of steps during centriole formation in *C. elegans*, from two mother centrioles (gray cylinders, with nine singlets of microtubules) to two pairs of centrioles, each containing one mother and one daughter centriole. Our work indicates that SPD-2 is recruited first (step 1). ZYG-1 recruitment occurs at about the same time and requires SPD-2 function (step 2). This is followed by recruitment of SAS-5 and SAS-6 (step 3). ZYG-1 at centrioles then diminishes, provided that SAS-5 and SAS-6 are present (step 4), after which SAS-4 is incorporated in the newly forming centriole (step 5).

(H) A pathway for centriole formation in *C. elegans*. Steps are as described in (G). Arrows indicate positive interactions. A negative interaction is also indicated. See text for details. Note that SPD-2 also plays a role in the maintenance of SAS-4 (see Figure 2), a function that does not go through the other proteins in the pathway, since SAS-4 in paternally contributed centrioles is not affected by depletion of SAS-5 or SAS-6 [7–9].

AIR-1 and γ-tubulin [5, 6]. Thus, SPD-2 lies upstream in the pathway for PCM assembly. Similarly, we found that SPD-2 is the upstream-most component among the five proteins essential for centriole formation. Therefore, SPD-2 plays a pivotal role in coordinating assembly of the two principal constituents of centrosomes. Perhaps SPD-2 acts in a manner analogous to scaffold proteins in signaling networks, which serve to localize and modulate kinases and their substrates [20]. In this scenario, ZYG-1 and its substrates may be brought together by SPD-2 during centriole formation. *spd-2* and *zyg-1* exhibit a strong genetic interaction [5, 6], compatible with the two components having a close relationship. Interestingly, we discovered that SAS-5 and SAS-are needed for the diminution of centriolar ZYG-1 during interphase. Whereas it remains to be determined whether diminution of centriolar ZYG-1 is important, it is tempting to speculate that this serves as a signal ensuring that SAS-5 and SAS-6 have been recruited before further steps can take place.

Conclusions

The centriole is a microtubule-based structure whose regulated duplication is crucial for proliferation control. The mechanisms underlying centriole formation represent an important open question in cell biology. Five proteins have been identified in *C. elegans* as being essential for this process: ZYG-1, SAS-4, SAS-5, SAS-6, and SPD-2. We contributed to unravel the full epistatic relationships between these five proteins, thus paving the way for a mechanistic dissection of their participation in centriole formation. Homologs of SPD-2 and SAS-6 are present in other metazoans [6, 8, 9], and inactivation of HsSAS-6 in human cells abrogates centrosome duplication [9]. Moreover, distant homologs may also exist for ZYG-1 and SAS-4 [3, 21]. Owing to such conservation, we expect the relationships unraveled in this study to be of broad significance.

Experimental Procedures

Experimental Procedures can be found in the Supplemental Data available online with this article.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and two figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/18/1844/DC1/.

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